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Hair cell induction from human induced pluripotent stem cells using a simple stepwise method

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ABSTRACT

Disease-specific induced pluripotent stem cells (iPS) cells are expected to contribute to exploring useful tools for studying the pathophysiology of inner ear diseases and to drug discovery for treating inner ear diseases. For this purpose, stable induction methods for the differentiation of human iPS cells into inner ear hair cells are required. In the present study, we examined the efficacy of a simple induction method for inducing the differentiation of human iPS cells into hair cells. The induction of inner ear hair cell-like cells was performed using a stepwise method mimicking inner ear development. Human iPS cells were sequentially transformed into the preplacodal ectoderm, otic placode, and hair cell-like cells. As a first step, preplacodal ectoderm induction, human iPS cells were seeded on a Matrigel-coated plate and cultured in a serum free N2/B27 medium for 8 days according to a previous study that demonstrated spontaneous differentiation of human ES cells into the preplacodal ectoderm. As the second step, the cells after preplacodal ectoderm induction were treated with basic fibroblast growth factor (bFGF) for induction of differentiation into otic-placode-like cells for 15 days. As the final step, cultured cells were incubated in a serum free medium containing Matrigel for 48 days. After preplacodal ectoderm induction, over 90% of cultured cells expressed the genes that express in preplacodal ectoderm. By culture with bFGF, otic placode marker-positive cells were obtained, although their number was limited. Further 48-day culture in serum free media resulted in the induction of hair cell-like cells, which expressed a hair cell marker and had stereocilia bundle-like constructions on their apical surface. Our results indicate that hair cell-like cells are induced from human iPS cells using a simple stepwise method with only bFGF, without the use of xenogeneic cells.

Key Words: inner ear hair cells, preplacodal ectoderm, otic placode, bFGF, pluripotent stem cell, disease-specific iPS cells

Highlights

1) Hair cell-like cells were induced from human iPS cells by a simple stepwise method.

2) Hair cell-like cells were induced from human iPS cells using no xenogeneic cells.

3) Treatment with bFGF induced otic placodal cells but had limited efficiency.

Abbreviations:

iPS cells, induced pluripotent stem cells; MSCs, mesenchymal stem cells; ES cells, embryonic stem cells; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; KSR, knockout serum replacement; PBS, phosphate-buffered saline; PFA, paraformaldehyde; BSA, bovine serum albumin; DAPI, 4', 6-diamino-2-phenyl-indole; SFEBq, serum-free floating culture of embryoid body-like aggregates with quick reaggregation; RA, retinoic acid; SHH, Sonic hedgehog

1. Introduction

The establishment of human induced pluripotent stem (iPS) cells has made it possible to generate pluripotent stem cells from adult somatic cells [24, 27], which has allowed the use of disease-specific iPS cells for various purposes. In particular, in the field of inner ears, disease-specific iPS cells may play important roles in revealing the pathophysiology and in drug discovery for inner ear diseases because of the difficulty in sampling inner ear tissues. To realize studies using inner ear disease-specific iPS cells, stable methods for the differentiation of human iPS cells into inner ear cells are required. Methods for the induction of hair cells or otic progenitors from mouse mesenchymal stem cells (MSCs) [8], human MSCs [4], mouse embryonic stem (ES) cells [9, 12, 18-20], mouse iPS cells [18, 25], and human ES cells [2, 21] have been reported. However, previously

reported methods have several issues, including the use of complex combinations of growth factors, inhibitors, xenogeneic cells such as chicken or mouse mesenchymal cells, or conditioned medium from murine stromal cells. For the screening of novel pharmacological agents, simple induction methods are ideal

Methods that mimic each step of inner ear development are desirable for inner ear induction of human iPS cells. In brief, the ectoderm differentiates into the neural ectoderm and nonneural ectoderm following the formation of the three germ layers [26]. Following this, rostral ectodermal cells next to the neuroectoderm differentiate into the preplacodal ectoderm [13] containing precursors for various sensory placodes, including the otic placode [7]. After otic placode induction from a region of the preplacodal ectoderm [6, 10], the otic placode invaginates and forms the otic vesicle by pinching off from the surface ectoderm [22]. Each area of the inner ear, including the endolymphatic duct, semicircular canal, utricle, saccule, and cochlea, is formed from the otic vesicle [5].

The primary step in mimicking inner ear development is the induction of the preplacodal ectoderm from human iPS cells. A recent study demonstrated that serum free N2/B27 medium induced spontaneous differentiation of human ES cells into the preplacodal ectoderm, unlike mouse ES cells that differentiate into neural cells under the same condition [11]. In the present study, we examined the potential of serum free N2/B27 medium to induce preplacodal ectoderm differentiation of human iPS cells.

The next step involves the differentiation of preplacodal ectodermal cells into the otic placode. It is well known that basic fibroblast growth factor (bFGF) plays a key role in otic induction [14, 16]. Therefore, we examined the efficacy of bFGF in otic placode induction of preplacodal cells derived from human iPS cells. Finally, we investigated the potential of our method for hair cell induction. For this purpose, after otic induction, cultured cells were incubated with serum free medium, and evaluated their fate.

2. Materials and methods

2.1. Human iPS cells

The human iPS cell line 201B7 [24] was maintained as described previously [15]. In brief, human iPS cells were maintained on SNL feeder cells treated with mitomycin-C (Kyowa Hakko Kirin Co, Ltd, Tokyo, Japan) in human iPSC medium consisting of Dulbecco's modified Eagle's medium (DMEM)/F-12 (Nacalai Tesque, Kyoto, Japan) supplemented with 20% knockout serum replacement (KSR; Invitrogen, Carlsbad, CA), 1% Glutamax (Invitrogen), 1% minimum essential medium nonessential amino acid solution (Wako, Osaka, Japan), 0.1 mM 2-mercaptoethanol (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 ng/mL recombinant human bFGF (Wako). Cells were passaged using a dissociation solution consisting of phosphate-buffered saline (PBS) containing 0.25% trypsin (Invitrogen), 1 mg/mL collagenase type IV (Invitrogen), 1 mM $CaCl_2$ (Wako), and 20% KSR.

2.2. Preplacodal ectoderm induction

We performed preplacodal ectoderm induction from human iPS cells according to the methodology described in a previous study that demonstrated spontaneous differentiation of human ES cells into the preplacodal ectoderm using a serum free N2/B27 medium [11]. Prior to induction, human iPS cells were cultured in SNL-conditioned medium containing 5 ng/mL bFGF on culture dishes coated with Matrigel (hESC-qualified Matrix Cat.No.354277, BD Biosciences, San Jose, CA). SNL-conditioned medium is derived from the supernatant of SNL feeder cells cultured in hiPSC medium for 24 h. Human iPS cells were dissociated into single cells with Accumax (ICT, San Diego, CA) and suspended in SNL-conditioned medium containing 5 ng/mL bFGF and 10 μM Rho kinase inhibitor, Y27632 (Wako) before plating at a density of 2×10^4 cells per well on cover slips (diameter, 12 mm) coated with Matrigel (Matrigel Matrix Growth Factor Reduced Cat.No.354230, BD Biosciences, San Jose, CA) in 24-well culture plates and incubation in SNL-conditioned medium. After 2 days, the medium was changed to serum free N2/B27 medium consisting of DMEM/F12 supplemented with 1xN2 (Invitrogen), 1xB27 (Invitrogen), 1% Glutamax (Invitrogen),

1% minimum essential medium nonessential amino acid solution (Wako), and 0.1 mM 2-mercaptoethanol (Invitrogen). Cells were cultured in serum free N2/B27 medium for 8 days. The medium was replaced every day. After preplacodal ectoderm induction, cells were examined by immunocytochemistry for SIX1 and E-CADHERIN that express in preplacodal ectoderm. And we calculated the proportion of SIX1- and E-CADHERIN-double positive cells in the total cell population.

2.3. Otic placode induction

For otic placode induction, the cells after preplacodal ectoderm induction were cultured in serum free N2/B27 medium containing 25 ng/mL bFGF for 15 days. Oshima *et al*. reported otic placode induction of mouse ES/iPS cells by 3-day treatment with bFGF [18]. Because the human developmental period is longer than the murine developmental period, we expected the induction period in human ES/iPS cells to also be longer than that in mouse ES/iPS cells. For example, opsin-positive cone photoreceptor induction periods are reported to be 28 days in mouse ES cells and 120–150 days in human ES cells [17]. Therefore, for otic placode induction, we elongated the bFGF treatment to 15 days, which is five times the duration of the method used by Oshima *et al*. [18]. The culture medium was replaced every day. Following otic placode induction, the cells were examined by immunocytochemistry for PAX2 and P63. The efficiency of induction was calculated as the proportion of PAX2-positive and P63-negative cells in the total cell population.

2.4. Hair cell induction

Human iPS cell derivatives including PAX2-positive cells were cultured in a serum free medium containing 1% liquid form Matrigel following otic placode induction according to a previously reported method [9] for hair cell induction. Human iPS cell derivatives including PAX2-positive cells were cultured in a serum free medium containing Matrigel which contains Advanced DMEM/F12 supplemented with 1xN2 (Invitrogen), 0.5% Glutamax (Invitrogen), and 1% Matrigel (Matrigel Matrix Growth Factor Reduced Cat.No.354230, BD Biosciences, San Jose, CA) for 48

days. Half of the culture medium was replaced every day. Following hair cell induction, cultured cells were examined by immunocytochemistry for Myosin 7a. The efficiency of induction was calculated as the proportion of Myosin 7a-positive cells in the total cell population. In addition, the surface morphology of cultured cells was examined by scanning electron microscopy.

2.5. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA) for 15 min and permeabilized with 0.2% Triton X-100 for 5 min. Cells were then treated with 1% bovine serum albumin (BSA; Wako) for 10 min at room temperature. Cells were incubated with primary antibodies overnight at 4°C. After washing with PBS, specimens were incubated with fluorescent-labeled secondary antibodies for 1 h at room temperature. Nuclei were labeled with 4′, 6-diamino-2-phenyl-indole (DAPI) (Invitrogen). F-actin was labeled with fluorescent-conjugated phalloidin. Signals were visualized using a BioRevo fluorescent microscope (Keyence, Osaka, Japan) or Leica TCS SP8 Confocal Laser Scanning Microscope (Leica Microsystems, Tokyo, Japan).

Primary antibodies used were anti-E-cadherin antibody (1:1000, BD Pharmingen), anti-Six1 antibody (1:1000, SIGMA), anti-P63 antibody (1:200, BIOCORE), anti-Neuronal class III β- tubulin antibody (1:1000, Covance Inc., Princeton, NJ), anti-Pax2 antibody (1:500, Covance), and anti-Myosin VIIa antibody (1:500, Proteus).

2.6. Scanning electron microscopy

After hair cell induction, to confirm the cell surface structure, cultured cells were observed by scanning electron microscopy. Cultured cells were fixed with 4% paraformaldehyde/2% glutaraldehyde in PB at 4°C for 4 h. Fixed cells were then dehydrated, dried using a critical point drying method, and coated with a thin layer of platinum palladium. Specimens were observed using the scanning electron microscope S-4700 (Hitachi Co., Tokyo, Japan).

3. Results

In the present study, we generated hair cell-like cells from human iPS cells using a stepwise method consisting of three steps: preplacodal ectoderm induction, otic placode induction, and hair cell differentiation (Fig. 1). We replicated the experiments three times independently for calculating the induction efficiency in each step. Cells were characterized at each induction step by immunocytochemistry.

3.1. Preplacodal ectoderm induction from human iPS cells

The first step was preplacodal ectoderm induction from human iPS cells by culturing in serum free N2/B27 medium for 8 days. After the first induction step, 97.4 ± 2.2% of the total cells expressed both SIX1 and E-CADHERIN that express in prepracodal ectoderm (Fig. 2A). This result indicates that human iPS cells were differentiated into an uniform cell population expressing the genes that express in preplacodal ectoderm.

3.2. Otic placode induction

In the second step, otic placode induction of the cell after preplacodal ectoderm induction was performed by culture with bFGF for 15 days. We examined the expression of PAX2, a marker for the otic placode, and P63, a marker for the epidermal cells, to determine the efficiency of induction. We defined PAX2-positive, P63-negative cells as otic placodal cells. PAX2-positive, P63-negative cells were observed in all three experiments (Fig. 2B). PAX2-positive cells formed a small number of compact clusters in each well on a culture plate. The proportion of PAX2-positive cells in the total cell populations was 0.047 ± 0.012%. PAX2- and P63-double-positive cells were not observed. In otic placode induction without bFGF, PAX2-positive cells were found in one out of three experiments and the proportion of PAX2-positive cells per total cells was 0.006%. Culture with bFGF generated otic placodal cells from the cells after preplacodal ectoderm induction, but with a low efficacy.

3.3. Hair cell induction

In the final step, human iPS cell-derivatives including PAX2-positive cells were cultured in a serum free medium containing Matrigel for 48 days. We examined the expression of the hair cell marker MYOSIN VIIa in cultured cells. MYOSIN VIIa-positive cells were found in all three experiments (Fig. 2C a-c). MYOSIN VIIa-positive cells formed a small number of compact clusters in a well per culture plate. The proportion of MYOSIN VIIa-positive cells in the total cells was 0.01 ± 0.008%. Cultured cells contained βIII tubulin-positive cells (Fig. 2C d-f).

The surface morphology of cultured cells was examined by scanning electron microscopy. Cultured cells included epithelial-like cells and neuron-like cells (Fig. 3a). A small number of epithelial-like cells had stereocilia-like protrusions at the center of the apical surface (Fig. 3b). Kinocilia-like structures were not present in stereocilia-like protrusions (Fig. 3c, d). The cells with stereocilia-like protrusions were found in two out of three experiments. Two wells of a 24-well culture plate were used for each experiment. Cells with stereocilia-like protrusions were observed in only one well in each experiment. The numbers of the cells with stereocilia-like protrusions in each well were 16 cells and 15 cells. These findings indicate that our method allows the induction of hair cell-like cells from human iPS cells, although the number is limited.

4. Discussion

In previous reports of hair cell induction from pluripotent stem cells using the stepwise method, cells were initially differentiated into ectodermal cells [18, 21]. Ectodermal cells have the potential to differentiate into various cell types, including neural cells, dermal cells, and preplacodal ectodermal cells. The preplacodal ectoderm is competence to develop sensory organs. Therefore, we used a stepwise induction method via the preplacodal ectoderm induction in the present study. Based on the results of a previous study of human ES cells [11], we cultured human iPS cells in DMEM/F12 supplemented with N2/B27 without the use of inhibitors. As a result, we obtained an uniform cell population expressing the genes that express in preplacodal ectoderm.

For otic placode induction, we supplemented only bFGF, which is known to play a key role in otic induction, in the culture media. Otic placodal cells were obtained in the present study; however, the efficiency of induction was not satisfactory. Compared with previous reports [18, 21], the efficiency of otic induction in the present study was extremely low. In previous reports, efficiencies of otic placode induction were 33.3% [21] in human ES cells, 29.8 ± 7.1% in mouse ES cells, and 19.6 ± 5.6% in mouse iPS cells [18]. These findings indicate that induction with bFGF alone is insufficient for otic placodal induction of the cells after preplacodal ectoderm induction. We, therefore, consider that manipulation of BMP signals should be applied likely in case of induction of human ES cells into hair cells [21].

Our simple stepwise method allowed the induction of hair cell-like cells from human iPS cells, although the efficacy of otic induction was poor. Improving the efficacy of otic induction step may increase the number of hair cell-like cells. In a previous study of hair cell induction from mouse ES cells [9], a modified serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq) method was employed [23]. In that study, treatment with a combination of a TGF-β inhibitor and BMP4, followed by treatment with a combination of a BMP inhibitor and bFGF, successfully induced differentiation toward otic fates [9]. The final induction of differentiation into hair cells was performed by culture in a serum free medium containing Matrigel, without the use of induction factors [9]. In the present study, using this method, otic placodal cells were cultured in the serum free medium containing Matrigel for hair cell induction. Some additional factors have the potential to improve the potency of induction to hair cells from otic placodal cells. Previous studies have revealed the roles of retinoic acid (RA) [1] and/or Sonic hedgehog (SHH) [3] signaling as inducers of the prosensory fate. In the future, we intend to examine the efficacy of hair cell induction by manipulation of the RA and/or SHH signaling pathway. After improving the efficacy of hair cell-like cell induction from human iPS cells, we intend to estimate the function of induced hair cell-like cells by electrophysiology.

In the present study, we generated hair cell-like cells from human iPS cells using a stepwise method with only one induction factor, bFGF, without the use of xenogeneic cells. For application of

drug discovery using disease specific iPS cells in the field of inner ears, further optimization of an otic placode induction step should be done.

Conflict of Interest Statement

The authors declare no competing financial interests.

Author contributions

H.O.: collection and assembly of data, data analysis and interpretation, and manuscript writing; D.S.: collection and assembly of data and manuscript writing; S.K.: Critical review; T.S.: Critical review; N.Y.: Critical review; J.I.: Financial support and Final approval of manuscript; T.N.: conception and design, data analysis and interpretation, and manuscript writing. All authors have approved the final article.

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Figure Legends

Fig. 1. Overview of the protocol for hair cell induction

The first step is preplacodal ectoderm induction from human iPS cells. For induction of the preplacodal ectoderm, human iPS cells were cultured for 8 days in serum free N2B27 medium. In the second step, the cells after preplacodal ectoderm induction were treated with bFGF for 15 days for otic placode induction. In the final step, human iPS cell-derivatives including PAX2-positive cells were cultured in a serum free medium containing Matrigel for 48 days for hair cell induction. PPE, preplacodal ectoderm; OP, otic placode; hiPSC, human induced pluripotent stem cells; SF, serum free N2B27; bFGF, basic fibroblast growth factor; MG, serum free containing Matrigel.

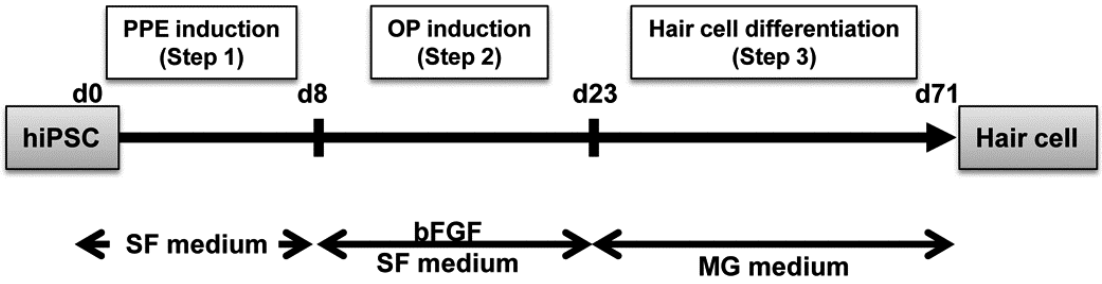

Fig. 2. Characterization of the cells at each induction step of hair cell induced from human iPS cells

Immunocytochemical analyses of cells at each induction step. (A) Following preplacodal ectoderm induction, cells demonstrated the expression of SIX1 (a) and E-CADHERIN (b). Scale bars, 50 μm. (B) Following otic placode induction, PAX2-positive (a) and P63-negative (b) cells were observed. PAX2-positive cells formed a small number of compact clusters per well on culture plates. Scale bars, 50 μm. (C) Following hair cell induction, cultured cells contained MYOSIN VIIa-positive cells (a) and βIII TUBULIN-positive cells (d). MYOSIN VIIa-positive cells formed a small number of compact clusters in a well per culture plate. Scale bars, 25 μm. Phalloidin shows f-actin. DAPI shows nuclei. βIII TUB, βIII TUBULIN; E-CAD, E-CADHERIN; MYO VIIa, MYOSIN VIIa; DAPI, 4′, 6-diamino-2-phenyl-indole .

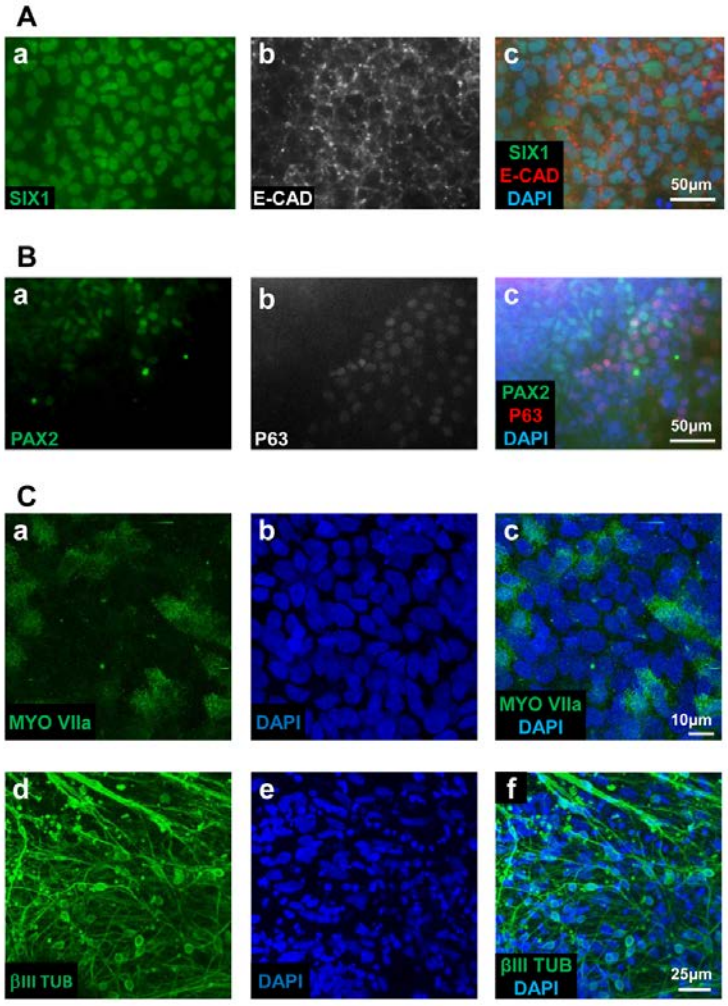

Fig. 3. Scanning electron micrographs of hair cell-like cells derived from human iPS cells

After hair cell differentiation, the surface morphology of cultured cells was examined by scanning electron microscopy. Cultured cells included epithelial cell-like cells (a, asterisk) and neuron-like cells (a, arrows). A small number of epithelial-like cells had stereocilia-like protrusion within the apical surface (b, white box). Kinocilia-like structures were not observed in stereocilia-like protrusions (c and d). Scale bars, 25μm, 10 μm, 5 μm, and 5 μm for a, b, c, and d, respectively.